6-27-2016

Specificity and ligand affinities of the cocaine aptamer: impact of structural features and physiological NaCl

Ashish Sachan
Iowa State University

Muslum Ilgu
Iowa State University

Aaron Kempema
Iowa State University

George A. Kraus
Iowa State University, gakraus@iastate.edu

Marit Nilsen-Hamilton
Iowa State University, marit@iastate.edu

Specificity and ligand affinities of the cocaine aptamer: impact of structural features and physiological NaCl

Abstract

The cocaine aptamer has been seen as a good candidate for development as a probe for cocaine in many contexts. Here, we demonstrate that the aptamer binds cocaine, norcocaine, and cocaethylene with similar affinities and aminoglycosides with similar or higher affinities in a mutually exclusive manner with cocaine. Analysis of its affinities for a series of cocaine derivatives shows that the aptamer specificity is the consequence of its interaction with all faces of the cocaine molecule. Circular dichroism spectroscopy and 2-aminopurine (2AP) fluorescence studies show no evidence of large structural rearrangement of the cocaine aptamer upon ligand binding, which is contrary to the general view of this aptamer. The aptamer's affinity for cocaine and neomycin-B decreases with the inclusion of physiological NaCl. The substitution of 2AP for A in position 6 (2AP6) of the aptamer sequence eliminated the effect of NaCl on its affinities for cocaine and analogues, but not for neomycin-B, showing a selective effect of 2AP substitution on cocaine binding. The affinity for cocaine also decreased with increasing concentrations of serum or urine, with the 2AP6 substitution blunting the effect of urine. Its low affinities for cocaine and metabolites and its ability to bind irrelevant compounds limit the opportunities for application of this aptamer in its current form as a selective and reliable sensor for cocaine. However, these studies also show that a small structural adjustment to the aptamer (2AP exchanged for adenine) can increase its specificity for cocaine in physiological NaCl relative to an off-target ligand.

Disciplines

Biochemistry, Biophysics, and Structural Biology | Chemistry

Comments

This is an article from Sachan, Ashish, Muslum Ilgu, Aaron Kempema, George A. Kraus, and Marit Nilsen-Hamilton. "Specificity and ligand affinities of the cocaine aptamer: impact of structural features and physiological NaCl." Analytical chemistry 88, no. 15 (2016): 7715-7723. DOI: 10.1021/acs.analchem.6b01633. Posted with permission.

analytical chemistry

Article

pubs.acs.org/ac

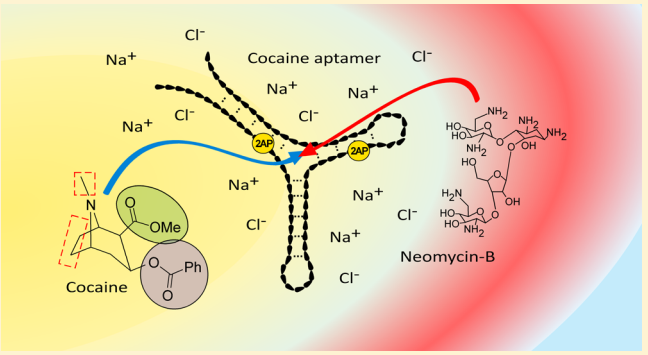
Specificity and Ligand Affinities of the Cocaine Aptamer: Impact of Structural Features and Physiological NaCl

Ashish Sachan,[†,∥] Muslum Ilgu,[§,⊥] Aaron Kempema,[‡,#] George A. Kraus,[‡] and Marit Nilsen-Hamilton*[,§,⊥]

[†]Interdepartmental Toxicology Program, [‡]Department of Chemistry, [§]Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, Iowa 50011, United States

Supporting Information

ABSTRACT: The cocaine aptamer has been seen as a good candidate for development as a probe for cocaine in many contexts. Here, we demonstrate that the aptamer binds cocaine, norcocaine, and cocaethylene with similar affinities and aminoglycosides with similar or higher affinities in a mutually exclusive manner with cocaine. Analysis of its affinities for a series of cocaine derivatives shows that the aptamer specificity is the consequence of its interaction with all faces of the cocaine molecule. Circular dichroism spectroscopy and 2-aminopurine (2AP) fluorescence studies show no evidence of large structural rearrangement of the cocaine aptamer upon ligand binding, which is contrary to the general view of this aptamer. The aptamer's affinity for cocaine and neomycin-B decreases with the inclusion of physiological NaCl. The substitution of 2AP for A in position 6 (2AP6) of the aptamer sequence eliminated the effect of NaCl on its affinities for cocaine and analogues, but not for neomycin-B, showing a selective effect of 2AP substitution on cocaine binding. The affinity for cocaine also decreased with increasing concentrations of serum or urine, with the 2AP6 substitution blunting the effect of urine. Its low affinities for cocaine and metabolites and its ability to bind irrelevant compounds limit the opportunities for application of this aptamer in its current form as a selective and reliable sensor for cocaine. However, these studies also show that a small structural adjustment to the aptamer (2AP exchanged for adenine) can increase its specificity for cocaine in physiological NaCl relative to an off-target ligand.



Probe specificity plays a vital role in analytical methods. For this reason, the reported high specificities of nucleic acid aptamers for their analytes and their adaptability to a variety of analytical platforms have created great interest in aptamers as selective probes for analytical chemistry. Consequently, the cocaine aptamer, initially reported to be highly specific for cocaine, has been the focus of much investigation.[1,2] Recently, this aptamer's specificity has been extended to include norcocaine and the off-target ligand quinine.[3−6] Since the cocaine-binding activity of the original MNS-4.1 aptamer was reported,[1] many functional variants of this aptamer have been reported. Here we report on the specificity, ligand binding, and structure of three sequence variants of the cocaine aptamer[2,7] and some of their 2-aminopurine (2AP)-substituted variants. Our results demonstrate that the aptamer discriminates the cocaine structure at all faces of the molecule. It also binds two important metabolites, norcocaine and cocaethylene, and several aminoglycosides with affinities similar to or higher than that for cocaine. Contrary to the predominant view of the binding mechanism of this aptamer, our results clearly show that the cocaine aptamer undergoes very minor structural changes on binding its ligand and that small variations in sequence can alter its ligand affinities. The results suggest that the most robust analytical applications of the cocaine aptamer are limited to analyte mixtures of low complexity with high concentrations of cocaine. The most effective sensor platforms are likely to be those that rely on properties other than large structural changes in the aptamer upon binding analyte.

MATERIALS AND METHODS

Cocaine Aptamers. Cocaine aptamers (Supporting Information Table S1) were from Integrated DNA Technologies (Coralville, IA, USA) or from Iowa State University DNA Facility (Ames, IA, USA) as standard desalted preparations. 2AP-modified aptamers were characterized by their λ_{ex} = 303 or 307 nm and λ_{em} = 370 or 371 nm. The aptamers are named according to their nt length and substituents and ending with #A to signify that the sequence has been identified as one of an aptamer series. Therefore, 38COC1−2AP6A is a 38 nt long cocaine aptamer with 2-aminopurine in position 6 and #1 in the series.

Biological Samples. Institutional Review Board (IRB) approved viral-tested human pooled urine was obtained from Bioreclamation Inc. (Westbury, NY, USA). The donors were verbally screened to be free of recreational drugs. Charcoal-

Received: April 26, 2016
Accepted: June 27, 2016
Published: June 27, 2016

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723

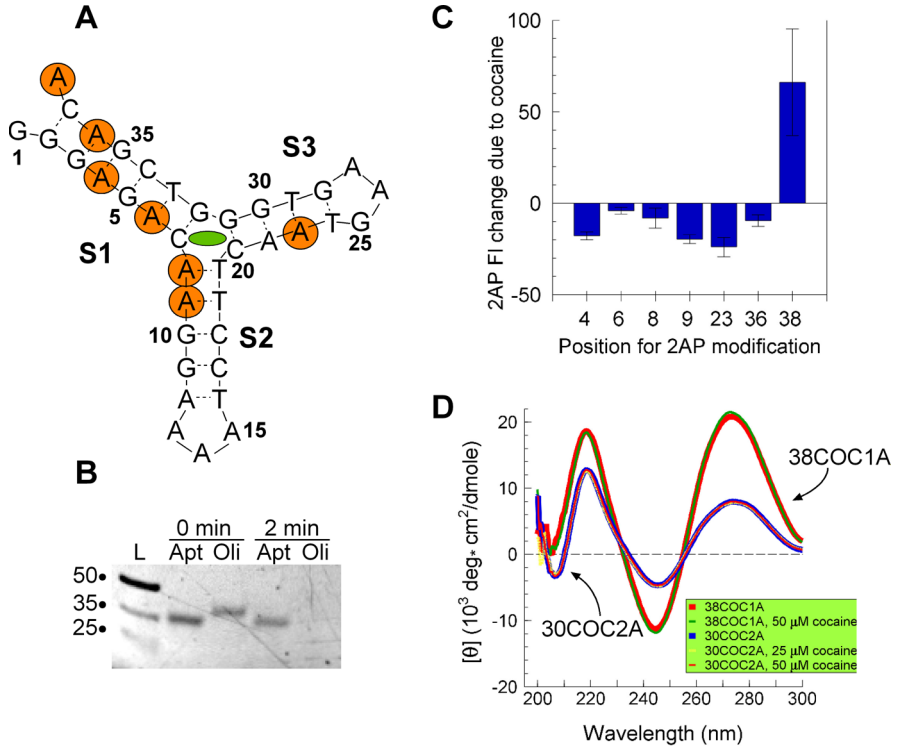


Figure 1. Minimal structural transition in the cocaine aptamer with ligand binding. (A) Proposed secondary structure of cocaine aptamer[6] with the positions of 2AP substitution identified by orange circles and cocaine by the green circle. S1, S2, and S3 are the stems. (B) The cocaine aptamer (Apt) or oligo40 (Oligo) was incubated for 0 or 2 min with 0.08 U/mL exo I and then the reaction stopped by bringing the solution to 1 mM EDTA. (C) A series of cocaine aptamers at 4 μM, each modified at a different position, were incubated with 200 μM cocaine. The fluorescence (λ_{ex} = 303 nm; λ_{em} = 371) was measured in the presence and absence of cocaine in buffer A. The difference (without cocaine subtracted from with cocaine) is plotted. (D) Circular dichroism spectra of 38COC1A and 30COC2A in 20 mM Tris, 5 mM KCl, pH 7.4, each in the presence and absence of cocaine.

stripped fetal bovine serum (FBS) was purchased from Sigma (St Louis, MO, USA).

Analytes. Cocaine (COC), norcocaine (NOR), cocaethylene (COE), benzoylecgonine (BZE), ecgonine methyl ester (EME), ecgonine ethyl ester (EEE), ecgonine (ECG), and anhydroecgonine methyl ester (AME) samples, dissolved in acetonitrile or methanol, were from Cerelliant (Round Rock, TX, USA). The analytes were separated from acetonitrile or methanol by initial dilution in deionized water followed by dehydration under vacuum during centrifugation (1400 rpm in an Eppendorf Vacufuge) for two cycles, each for 45 min at 60 °C. Protocols for synthesizing other cocaine analogues (6,7-dehydrococaine, 6,7-dehydrococaine-*tert*-*N*-BOC, 6,7-dehydrobiotinylcocaine, 6,7-dehydronorcocaine, and 6,7-bis(4-methylthiobenzoyloxy)cocaine) are in the Supporting Information (Figures S1 and S2). Neomycin-B, paromomycin, and ribostamycin were purchased from Sigma-Aldrich.

Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) experiments were performed using a VP-ITC isothermal titration calorimeter (Microcal, Inc., Northhampton, MA,USA) or NanoITC (TA Instruments, New Castle, DE, USA). The cocaine in the syringe and the cocaine aptamer in the cell were in buffer A (20 mM Tris, pH 7.4, 140 mM NaCl, 5 mM KCl) or buffer B (buffer A lacking NaCl) with the inclusion of urine or serum where indicated. All buffer pHs were measured at room temperature (23−24 °C) after their complete formulation.

Fluorescence Measurements. Fluorescent spectra were obtained using a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) with samples in a quartz cuvette at λ_{ex} = 303 or 307 nm and an emission scan of 340−585 or 320−450 nm, with a 5 or 10 nm slit. The PMT voltage was set to high. The maximum emission peak at 371 nm for each spectrum was selected for data analysis. OriginPro 8.6 was used to determine dissociation constants from the data obtained by steady state fluorescence quenching.

Circular Dichroism. Circular dichroism (CD) measurements were performed in a 0.1 cm path-length quartz cell with a Jasco J-710 spectropolarimeter at 25 °C with aptamers in 5 mM KCl, 20 mM Tris, pH 7.4. Data were collected from 200 to 300 nm with a scanning speed of 50 nm/min, resolution of 0.2 nm, bandwidth of 1.0 nm, sensitivity of 20 mdeg, and time response of 8 s. CD spectra (an average of results from two independently prepared aptamer solutions) are presented as the mean residue ellipticity, $[\theta]$ (deg·cm^2/dmol) vs wavelength (nm). CD data were analyzed by the program JFIT.

Gel Electrophoresis. Samples of cocaine aptamer incubated with or without exonuclease I (New England BioLabs, Ipswich, MA, USA) were resolved by nondenaturing polyacrylamide gel electrophoresis (PAGE), run for 3 h at constant 150 V in 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0. Acrylamide:bis(acrylamide) (37.5:1) was used to prepare the 15% acrylamide gel.

Statistical Analysis. Where indicated, statistical analyses were performed by an independent-samples two-tailed *t* test.

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723

Table 1. Specificity of the Cocaine Aptamer

	K_d + std dev (μM)				
cocaine analogue	38COC1A	36COC3A	38COC1−2AP23A		38COC1−2AP6A
cocaine	15 ± 6.2	20 ± 3.6	15 ± 2.8	28 ± 13[a]	9.2 ± 2.1
cocaethylene	9.8 ± 5.6	15 ± 2.4	11 ± 2.6	ND	6.5 ± 0.74
norcocaine	13 ± 3.0	12 ± 4.6	9.3 ± 0.75	ND	5.2 ± 0.87
ecgonine	NB	NB	NB	ND	73, NB[b]
6,7-dehydronorcocaine	56 ± 23	80 ± 17	ND	ND	ND
6,7-dihydroxycocaine	95, NB[b]	ND	ND	ND	ND

[a]The affinities of two cocaine aptamers and the 38COC1A aptamer with 2AP substituted for A at position 6 (38COC1−2AP6A) or 23 (38COC1−2AP23A) were determined by ITC for cocaine and cocaine analogues with the exception of one value, which was determined by 2AP steady state fluorescence quenching. All values are the average of at least two independent estimates with the values for cocaine being the averages of three to five independent estimates. [b]Two independent estimates for which one gave no binding and the other low affinity binding. Standard deviations are for independent estimates. The following analogues were also tested with two independent trials and found not to be bound by the aptamer: anhydroecgonine, benzoylecgonine, 6,7-bis(4-methylthiobenzoyloxy)cocaine, cocaine-*tert*-*N*-biotin, 6,7-dehydrococaine-*tert*-*N*-Boc, ecgonine ethyl ester, and ecgonine methyl ester. NB, no binding; ND, not determined.

RESULTS AND DISCUSSION

Structural Changes in the Cocaine Aptamer Accompanying Cocaine Binding. When bound to cocaine, the cocaine aptamer is believed to have a folded structure that consists of three stems (S1, S2, and S3) intersecting at a three-way junction (Figure 1A). It has frequently been represented as shifting from a largely unfolded structure (with only S2 intact) to one that is folded with bound cocaine.[1] This original model of a structure-switching aptamer, although pervasive in the literature, was shown by EPR and NMR analysis to be incorrect for S3, which was demonstrated to remain intact in the absence of cocaine for all versions of the cocaine aptamer tested.[7,8] However, in a version of the aptamer with a 3 bp S1 stem, this stem was reported as unstructured in the absence of cocaine.[8] The shorter stem also decreases the aptamer affinity for cocaine to 28−45 μM (K_d), which is 4−5-fold lower than for a version of the aptamer with a longer stem.[7] An examination of the literature from the past 3 years shows that the vast majority of sensors developed to incorporate the intact cocaine aptamer included the longer S1 stem,[5,9−22] with occasional comparisons of the short and long S1 aptamers.[5,16] Sensors have also been infrequently reported based on intact short stem cocaine aptamers.[23,24]

To understand the extent of structural unfolding that occurs with the cocaine aptamer, we first examined the most frequently employed aptamer with a longer S1 (38COC1A) that has been proposed to form a helix in the absence of cocaine.[7,8] If S1 in 38COC1A exists as a helical structure in the absence of cocaine, it should resist degradation by 3′ exonuclease I (Exo I), which is specific for single-stranded DNA. The control oligonucleotide for Exo I activity was a 40 nt oligomer (Oligo40) for which no significant structure was predicted by MFold.[25] Whereas Oligo40 was completely degraded after 2 min with 0.08 U/μL Exo I, 38COC1A was unaffected (Figure 1B). Therefore, in the absence of cocaine, 38COC1A appears to be completely folded including the S1 stem.

To determine the extent to which the cocaine aptamer structure shifts with cocaine binding, we examined a series of cocaine aptamer derivatives each with a single adenine (A) substituted by 2AP at positions 4, 6, 8, 9, 23, 36, and 38 (sequences in Table S1). Stacking of 2AP with other bases quenches the fluorescence of this purine analogue.[26] Small changes in fluorescence were observed to accompany cocaine binding to aptamer variants containing 2AP at various positions throughout the molecule with all but one change being a lower fluorescence in the presence of cocaine compared with the apo-aptamer (Figure 1C). The small change in fluorescence yield for the 3′ base (55−59% of that for the free 2AP base) suggests a small structural adjustment. This result is consistent with the small change in fluorescence quenching of a 3′ terminal fluorophore when the 5′ end was labeled with a matched quencher.[27]

We also examined the effects of cocaine on the secondary structures of 30COC2A (S1 = 3 bp) and 38COC1A (S1 = 6 bp) by CD spectroscopy (Figure 1D). The results clearly support the conclusion that the aptamer does not undergo a large change in secondary structure on binding cocaine even when the S1 stem is short.

Affinity of the Cocaine Aptamer and 2AP Derivatives for Cocaine. Although 2AP is considered a silent replacement of A because it also base-pairs with thymine,[28] it differs from A in some of its base-pairing options and its presence introduces small perturbations in the DNA helix structure.[29] Small local perturbations due to the replacement of A by 2AP could be amplified to larger changes in tertiary structure that might alter the aptamer's interactions with ligands. To investigate if the incorporation of 2AP into the cocaine aptamer changed its structure, we determined the affinities for cocaine of the aptamer and each of its 2AP derivatives. A large range of values (1 nM to 1 mM) has been reported for the affinity of the cocaine aptamer for cocaine.[2,4,8,27,30−36] This variation may be due to a combination of differences in aptamer sequence and assay conditions used in these studies. Consequently, we determined the affinities for cocaine of several versions of the cocaine aptamer by ITC under the same conditions (Table 1 and Figure S3). All tested aptamer variants displayed K_ds for cocaine of 10−20 μM.

As a control to establish that the aptamer DNA structure is critical for cocaine binding, we tested the ability of 38COC1A-RNA to bind cocaine. This oligonucleotide has the same base sequence as 38COC1A, but the H in the 2′ positions are replaced by OH and the methyl group on the thymidine is absent in the uracil substitution. These small structural changes resulted in an oligonucleotide that does not bind cocaine (Figure S4), thereby demonstrating the importance of structure over sequence in the cocaine aptamer.

For some 2AP substitutions the changes in fluorescence due to cocaine binding were sufficiently large to be used as monitors of cocaine binding (Figure 1C). With 2AP

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723

fluorescence as the readout (Figure 2A), we determined the K_d of 38COC1−2AP23A to be not significantly different from the

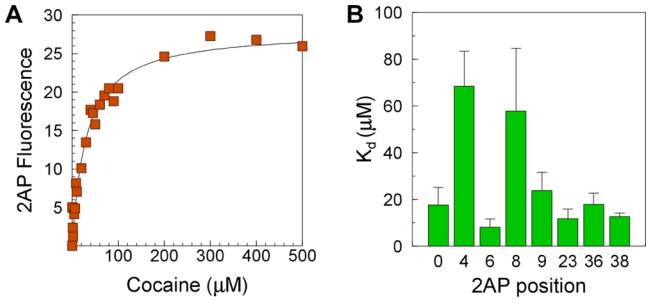


Figure 2. 2AP scan for structural changes in the cocaine aptamer with cocaine binding. (A) Percent fluorescence quenching (at 371 nm) of 38COC1−2AP23A with increasing cocaine in buffer A. The measured K_d was 30 μM. (B) K_ds of aptamers with site-specific 2AP modifications for cocaine in buffer A determined by ITC. Data for the unmodified cocaine aptamer are plotted in the 0 2AP position.

K_d obtained by ITC (Table 1). Nucleic acids with 2AP substituted for A in a specific position have been used in many studies to interrogate changes in nucleic acid structure with the implicit assumption that 2AP substitutions do not alter the nucleic acid structure.[28,37−39] We tested this assumption for the cocaine aptamer and found that the affinities for cocaine of most 2AP-substituted aptamers were comparable with that of the unsubstituted aptamer (Figure 2B). However, 2AP substitution in two positions (4 and 8) decreased the affinity compared with the unsubstituted aptamer (P = 0.0006 and 0.01 for positions 4 and 8, respectively). Excluding the results from these two substituted aptamers, which are likely altered in structure by 2AP substitution, our results are consistent with the conclusion that all stems of the apo-aptamer (ligand-free) are folded in the absence of cocaine and that very small structural changes occur throughout the molecule with cocaine binding (Figure 1C,D). These results are consistent with the reports of others.[5,7,8]

To test for independent effects of cocaine on 2AP fluorescence, the structures of the 38COC1−2AP derivatives were distorted by annealing them with a complementary 20 nt oligonucleotide (cDNA(1−20)) that covers the presumptive cocaine-binding pocket. These hybrid molecules showed no change in 2AP fluorescence upon the addition of cocaine, cocaine metabolites, or neomycin-B (Figure S5). Analyses by ITC showed that these distorted 38COC1A hybrids were not functional aptamers (Figure S6).

These results suggest that, rather than being explained by a large change in secondary structure as originally proposed for the cocaine aptamer, the ligand-driven changes in spectral characteristics,[27] electrochemical signals,[40,41] and bending of aptamer-decorated microcantilevers[42] are likely to be reporting on a change in its tertiary structure as was recently demonstrated by small-angle X-ray scattering analysis of the effect of cocaine on the aptamer's shape.[5]

Specificity of the Cocaine Aptamer for Cocaine and Related Compounds. Because specificity is an important feature that identifies aptamers as desirable probes for detecting analytes, we examined the ability of the 38COC1A to bind a range of cocaine analogues, including the major cocaine metabolites, for which modifications are located variously around the core molecule. Our results show that the aptamer binds cocaine, norcocaine, cocaethylene, and 6,7-dehydronorcocaine with affinities in the range of 10−56 μM and to 6,7-dihydroxycocaine with lower affinity, but does not bind any other analogue tested (Table 1). Similar results were found by ITC and steady state 2AP fluorescence quenching (Figure 3A) and also for the 36COC3A aptamer.

Evaluation of the molecular features of analogues that bound and did not bind the cocaine aptamer suggests that this aptamer fully surrounds cocaine in the aptamer−ligand complex (Figure 3B and Table 1). The results show that addition of molecular bulk (even a methyl group) to the N8 position of cocaine decreases the aptamer affinity (cf. norcocaine and cocaine; Table 1). The C2-linked methyl or ethyl esters and the phenylester at C3 promote aptamer binding (cf. cocaine and anhydroecgonine methyl ester). Cocaine derivatives containing either a double bond or hydroxyl groups at positions C6 and C7 bound poorly, which showed that the C6−C7 face of the cocaine molecule also interacts with the aptamer. These results show that all faces of cocaine bind to the aptamer and suggest that specificity for cocaine may result from the ligand being buried in the DNA as has been observed for other aptamer−ligand interactions.[43]

Originally reported to bind cocaine and neither benzoylecgonine nor ecgonine methyl ester,[2] the aptamer was later shown to also bind norcocaine.[4,6] We show here that it also binds cocaethylene with an affinity similar to that for cocaine and more tightly with norcocaine. Broadening the specificity of the cocaine aptamer to cocaethylene, a cocaine metabolite associated with ethanol consumption[44,45] is noteworthy because drugs are often consumed in combination with alcohols.

Heterologous Ligands Bound by the Cocaine Aptamer. To further explore the ligand selectivity of the cocaine aptamer, we examined its ability to bind aminoglycosides, a class of antibiotics that recognize many RNA structures.[46] We report for the first time that the cocaine aptamer binds the three tested aminoglycosides with affinities in the same concentration range as those for cocaine and metabolites (Table 2). These results are consistent with our observations that the aptamer exists in a primarily folded state when not bound to cocaine such that it can capture aminoglycosides, but they are unexpected because aminoglycosides generally bind to RNA and not DNA. DNA aptamers have recently been isolated for aminoglycosides, but their structures are unknown.[47,48] A-DNA is reported to bind aminoglycosides,[49] but our CD spectra clearly identify the cocaine aptamer structure as B-DNA (Figure 1D).

Effect of NaCl on Cocaine Aptamer Affinities. The affinities and specificities of aptamer−analyte interactions can be influenced by salt composition, which can vary between biological samples.[50,51] The effect of physiological (140 mM) NaCl was to decrease the affinities of 38COC1A for cocaine and neomycin-B by 4-fold and 2-fold, respectively (Figure 4A). This consistency between ligands suggests a global effect of NaCl on aptamer structure rather than an effect on specific ligand−aptamer contacts. Several secondary structures were predicted by MFold[25] of 38COC1A in the presence of 5 and 140 mM NaCl (Figure 4D), but not those originally proposed for this aptamer (Figure 1A). These folding predictions, in combination with the evidence (Figures 1B−D) that binding cocaine results in little change in secondary structure of the aptamer, suggest that the structural stability of 38COC1A derives mainly from tertiary structural interactions that may

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723

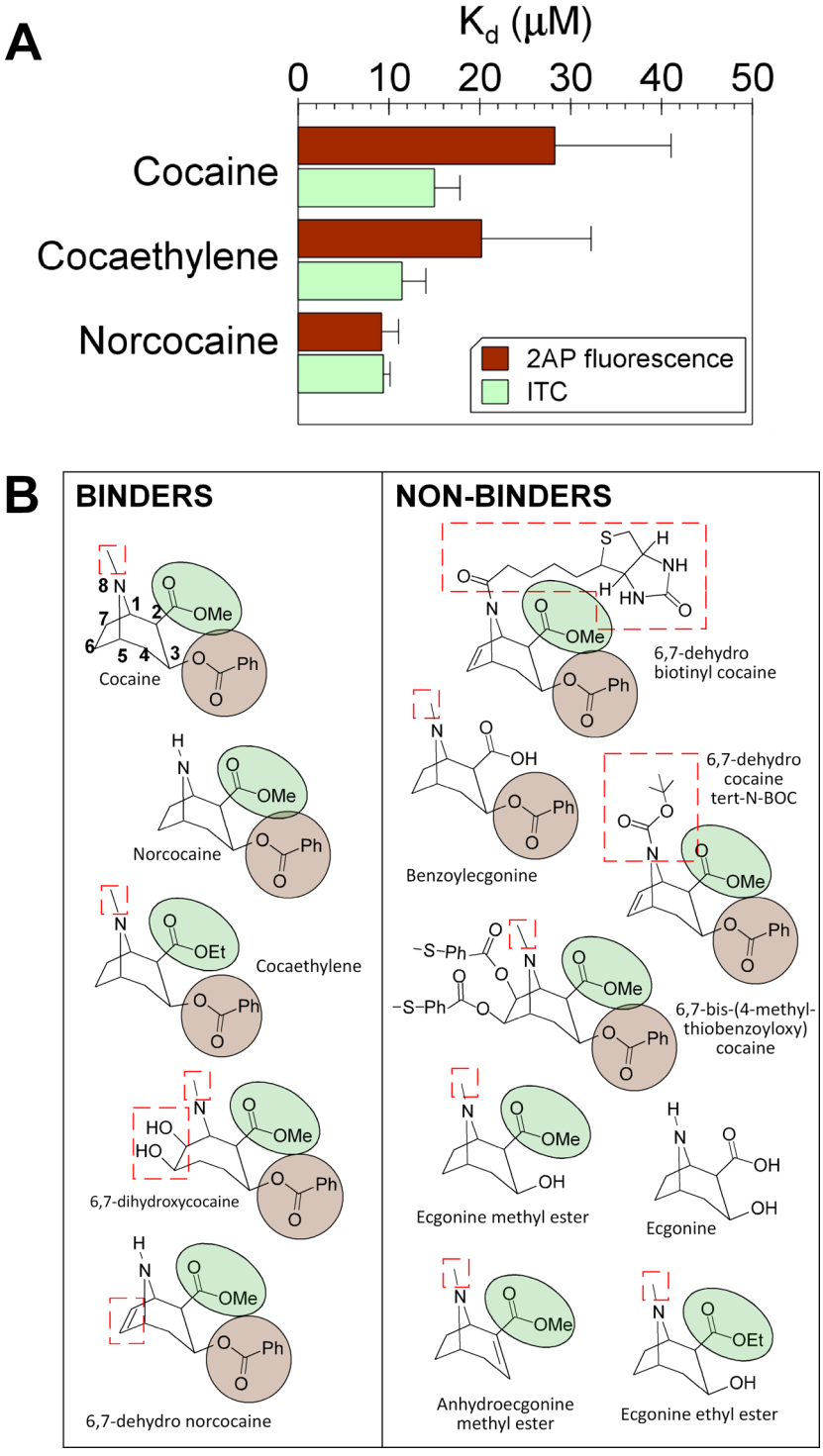


Figure 3. Specificity of the cocaine aptamer for cocaine analogues and metabolites. (A) 38COC1−2AP23A affinity for cocaine and its metabolites showing interassay variabilities for fluorescence and ITC measurements. (B) Functional groups in cocaine that promote 38COC1A aptamer recognition are circled. Moieties that decrease affinity for cocaine are surrounded by rectangles with dashed borders.

impose a consistent base-pairing pattern on the aptamer. The homogeneity of folding is evidenced by (1) *N* values of ∼1.0 from ITC measurements of binding to cocaine, norcocaine, and cocaethylene, (2) a single form on nondenaturing electrophoresis (Figure 1C), and (3) the CD spectra (Figure 1D) of this aptamer. The effect of NaCl on the aptamer affinity might be explained by the ability of monovalent cations to destabilize folded DNA structures.[52−55] If so, a related aptamer with a more stable secondary structure might not be affected by NaCl concentration. We tested this hypothesis with 36COC3A, which is predicted by MFold[25] to adopt one of two structures in the presence of both 5 and 140 mM NaCl, each with three-way junctions (Figure 4E). As for 38COC1A, affinities of 36COC3A for cocaine, cocaethylene, and norcocaine were decreased by the inclusion of 140 mM NaCl (Figure 4B). This suggests that, although the effect of NaCl on aptamer structure

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723

Table 2. Affinities for Aminoglycosides of the Cocaine Aptamer[a]

aminoglycoside, aptamer	analysis method	K_d ± std dev (μM)
neomycin-B, 38COC1A	ITC	4.5 ± 0.6
neomycin-B, 38COC1−2AP23A	fluorescence quenching	15 ± 4.0
paramomycin, 38COC1A	ITC	10 ± 3.4
ribostamycin, 38COC1A	ITC	75 ± 4.4

[a]The cocaine aptamers 38COC1A and 38COC1−2AP23A, for which the sequences can be found in Table S1, were tested for affinity for each of three aminoglycosides by ITC or 2AP fluorescence quenching.

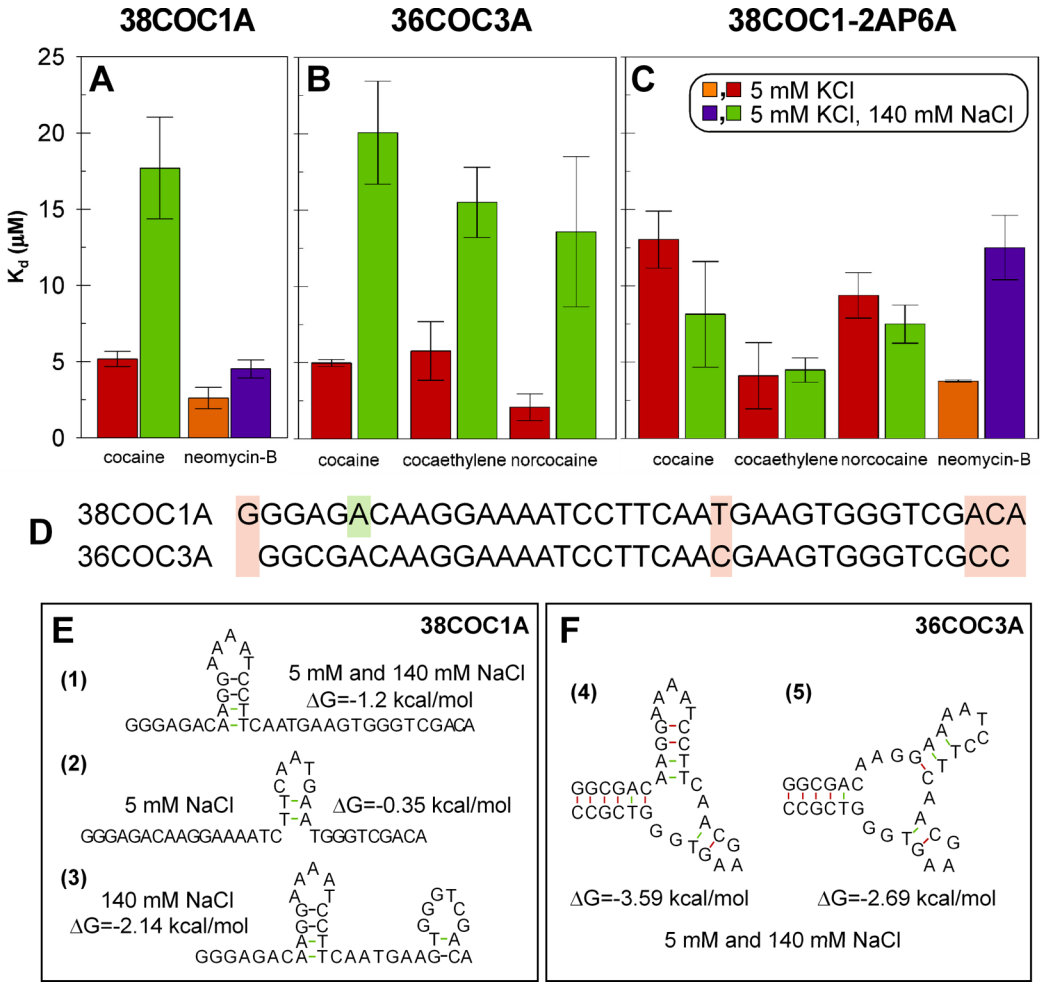


Figure 4. Effect of NaCl on the binding affinities of cocaine aptamers. Affinities of 38COC1A (A), 36COC3A (B), and 38COC1−2AP6A (C) for cocaine, cocaethylene, norcocaine, and neomycin-B in the presence of 5 mM KCl (red and orange) or 5 mM KCl, 140 mM NaCl (green and purple). The sequences of the two aptamers are compared in (D) with the differences identified in red shading and the position of the 2AP in 38COC1−2AP6A identified with green shading, Secondary structure predictions are given for 38COC1A (E) and 36COC3A (F) at 24 °C in 5 mM or 140 mM NaCl. No folding was predicted for either aptamer in the absence of NaCl. Although K^+ and Na^+ may interact differently with DNA, the MFold web server provides no option for folding in the presence of KCl. Therefore, 5 mM NaCl was used as a surrogate for 5 mM KCl.

is likely to be global, it is not driven by the strength of the S1 stem.

In contrast with 38COC1A, there was no effect of NaCl on cocaine affinity of the variant aptamer, 38COC1−2AP6A with 2AP substituted for A in position 6 (Figure 4C). However, the affinity of 38COC1−2AP6A for neomycin-B was decreased more by NaCl than seen with 38COC1A. The 2AP6 substitution also changed the specificity of 38COC1A from norcocaine = cocaethylene > cocaine to cocaethylene > norcocaine > cocaine (Table 1 and Figure 4C).

The selective effect of 2AP on the sensitivity to NaCl of the aptamer for cocaine binding compared with neomycin-B might be because neomycin-B binds in a distinct region of the aptamer from cocaine allowing a local effect on the cocaine-binding pocket to selectively alter the aptamer response to NaCl for cocaine. To establish if neomycin-B and cocaine bound the same locations on the aptamer, we determined the affinity by ITC when the two compounds were titrated separately into the aptamer or as a stoichiometric mixture. Separately and in combination, the ligands bound with estimated N values of ∼1 (Table 3 and Figure S7). The measured K_d for the combination of ligands was the average of the K_ds calculated from titrations with each ligand separately. These results demonstrate that neomycin-B and cocaine bind

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723

Table 3. Interaction of the Cocaine Aptamer with Cocaine and Neomycin-B[a]

ligand	K_d (μM)	N
cocaine	15 ± 6.2 (5)	0.7 ± 0.4 (4)
neomycin-B	4.5 ± 0.6 (2)	1.0 ± 0.1 (2)
av of cocaine and neomycin-B	11	0.9
cocaine plus neomycin B	11 ± 0.9 (2)	1.1 ± 0.01 (2)

[a]The affinities and N values of 38COC1A for cocaine and neomycin-B and an equimolar mixture of cocaine and neomycin-B are shown in addition to the average values for K_d and N for cocaine and neomycin-B. Shown also are the standard deviations and the number of independent estimates (in parentheses) for each value. The data for K_d for cocaine and neomycin-B are also shown in Tables 1 and 2, respectively. Representative titrations are shown in Figure S6.

to the same site on the molecule, thereby ruling out two binding sites as an explanation for the differential effect of the 2AP substitution on the NaCl sensitivity of affinities for cocaine and neomycin-B.

Effect of Biological Fluids on Cocaine Aptamer Affinities. Cocaine is excreted in urine as cocaine (1−9%), benzoylcocaine (35−45%), ecgonine methyl ester (32−49%), and ecgonine.[56,57] Of the metabolites that the aptamer binds, only norcocaine (~60 nM) is present in urine samples.[58] Individuals who have consumed alcohol also excrete cocaethylene.[44] Although human plasma and saliva can also contain cocaine at average concentrations of 3 and 31 μM, respectively,[59] urine is the biological matrix of choice because it can be obtained noninvasively and contains detectable concentrations of cocaine and its metabolites.[60] In the presence of 55% serum, the affinity of the 38COC1A aptamer for cocaine was very low and difficult to measure, whereas 38COC1−2AP6A and 38COC1−2AP23A retained their abilities to bind cocaine. Therefore, studies of the effects of urine and serum on the binding affinity for cocaine were limited to 38COC1A modified with 2AP substitution for A at positions 6 and 23, respectively (Figure 5). The cocaine affinity of 38COC1−2AP6A was much less affected by urine than by serum. In contrast to 38COC1−2AP6A, 38COC1−2AP23A demonstrated a much larger decrease in affinity for cocaine with increasing urine concentration. This observation that the placement in the aptamer sequence of a modified base can alter its performance in particular analyte matrices is consistent with the notion that the 2AP substitution can affect tertiary structure.

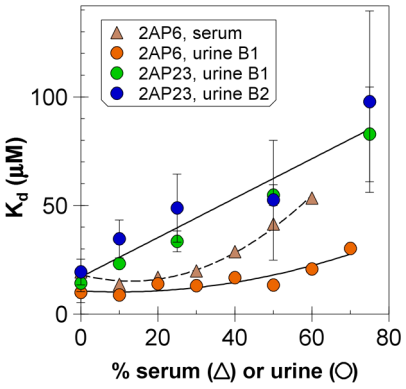


Figure 5. Effects of biological fluids on the binding affinities of cocaine aptamers. Aptamers modified at positions 6 (38COC1−2AP6A) and 23 (38COC1−2AP23A) with 2-aminopurine were tested by ITC for their binding affinities for cocaine in the presence of a range of concentrations of urine (●, ○) or fetal bovine serum (△). Two batches (B1, B2) of urine were tested.

The aptamer affinities shown in Figure 5 were determined with the NaCl concentration adjusted to the same value at all concentrations of biological fluids tested. Thus, these observations suggest that, in addition to NaCl, other components of these biological fluids also decrease the aptamer's affinity for its ligands. In a practical sense, these effects on K_d dampen the response of the aptamer to cocaine in these fluids and result in an underestimate of the cocaine concentration unless standard samples are dissolved in the equivalent fluid for analysis.

One concern for the accuracy of affinity values determined in the presence of biological fluids is the possible influence of cocaine degradation during the analysis period. The reported half-lives for cocaine in plasma and urine are approximately 2 and 9 h, respectively.[61,62] Although instability of cocaine in these biological fluids may have some impact on the measured dissociation constants, the observed decreases in affinity are inconsistent with a conclusion that ligand decay is the major factor influencing the measured affinity. First, at the end of a 2 h ITC run and with the reported decay rates, cocaine would be at a minimum 50% (assuming the same decay rate in serum as for plasma) or 85% (in urine) of the initial concentration, which would result in a maximum 2-fold increase in apparent K_d. But, the observed decreases in K_d are up to 5-fold of the control. Second, were the degradation of cocaine in urine responsible for the increased affinity, both 2AP-modified aptamers would be similarly affected in their affinity with incremental changes in urine concentration. But, with the same batch of urine, the pattern and extent of change in K_d is different for 38COC1−2AP6A and 38COC1−2AP23A (Figure 5).

■ CONCLUSIONS

Having evaluated the affinity of the cocaine aptamer for a broad range of cocaine metabolites and synthetic derivatives, we conclude that the aptamer interacts with all faces of the cocaine molecule. Despite this evidence that the cocaine is buried in the binding pocket, there are several troublesome off-target ligands that bind with similar or higher affinities (cocaethylene, norcocaine, aminoglycosides, and quinine),[3−6] which complicate the application of this aptamer in a cocaine sensor.

Although it is common for aptamers to respond to divalent cations such as Mg^{2+}, we have found that the cocaine aptamer is sensitive to NaCl. The results suggest that the effect is structural and the substitution of 2AP for A in position 6 of 38OC1A abrogates the effect of NaCl on the affinity for cocaine and its analogues but not for neomycin-B. Thus, NaCl lowers the aptamer affinity for cocaine but raises its specificity for cocaine over neomycin-B.

These results suggest that selective 2AP substitution of the cocaine aptamer might be a route to increasing its specificity and decreasing its responsiveness to environmental factors and biological matrix components. Additional structural stability required for good performance might be achieved by choosing the appropriate platform for application. For example, the observation that molecular crowding stabilizes nucleic acid structures[63,64] suggests optimal performance on platforms in which the aptamer is densely arrayed. At least three immobilized aptamers (cocaine,[42] thrombin, and Lcn2[65])

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723

demonstrated higher affinities for their ligands when they were immobilized compared with when they were in solution.

Its low affinity for cocaine, the effect of biological fluids on cocaine affinity, and the presence of several off-target ligands with similar affinities limit the application of this aptamer to the identification of cocaine and some of its analogues when they are present at high concentrations, in samples of known composition, in conjunction with tests for off-target ligands and standard samples in the same matrix as the analyte. However, the observation that aptamer affinity and specificity can be altered by selective substitution of 2AP for A in the DNA sequence suggests future opportunities for engineering this aptamer for higher affinity and specificity and less responsiveness to matrix components and salt compositions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b01633.

Oligonucleotides used, syntheses of cocaine-*tert*-*N*-biotin and 6,7-dimethylthiobutylacetylcocaine, and isothermal titration calorimetry procedures and results (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: marit@iastate.edu.

Present Addresses

[‖]Toxam Inc., Guelph, ON, Canada N1G 0B3.

[⊥]Aptalogic Inc., Ames, IA 50014, USA.

[#]Chemistry, Stemcentrx Inc., South San Francisco, CA 94080, USA.

Notes

The authors declare the following competing financial interest(s): M.N.-H is CSO of Aptalogic Inc. (API). M.I. is an employee of API and did part of the work described in this article while employed by the company.

■ ACKNOWLEDGMENTS

Financial support for this study was provided by the National Institute of Justice (Award No. 2008-DN-R-038) and Aptalogic Inc., Ames (API).

■ REFERENCES

(1) Stojanovic, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2001**, *123*, 4928−4931.

(2) Stojanovic, M. N.; Landry, D. W. *J. Am. Chem. Soc.* **2002**, *124*, 9678−9679.

(3) Sharma, A. K.; Heemstra, J. M. *J. Am. Chem. Soc.* **2011**, *133*, 12426−12429.

(4) Sharma, A. K.; Kent, A. D.; Heemstra, J. M. *Anal. Chem.* **2012**, *84*, 6104−6109.

(5) Reinstein, O.; Yoo, M.; Han, C.; Palmo, T.; Beckham, S. A.; Wilce, M. C.; Johnson, P. E. *Biochemistry* **2013**, *52*, 8652−8662.

(6) Slavkovic, S.; Altunisik, M.; Reinstein, O.; Johnson, P. E. *Bioorg. Med. Chem.* **2015**, *23*, 2593−2597.

(7) Neves, M. A.; Reinstein, O.; Johnson, P. E. *Biochemistry* **2010**, *49*, 8478−8487.

(8) Cekan, P.; Jonsson, E. O.; Sigurdsson, S. T. *Nucleic Acids Res.* **2009**, *37*, 3990−3995.

(9) Zheng, D.; Zou, R.; Lou, X. *Anal. Chem.* **2012**, *84*, 3554−3560.

(10) Porchetta, A.; Vallée-Bélisle, A.; Plaxco, K. W.; Ricci, F. *J. Am. Chem. Soc.* **2012**, *134*, 20601−20604.

(11) Yan, L.; Zhu, Z.; Zou, Y.; Huang, Y.; Liu, D.; Jia, S.; Xu, D.; Wu, M.; Zhou, Y.; Zhou, S.; Yang, C. J. *J. Am. Chem. Soc.* **2013**, *135*, 3748−3751.

(12) Nie, J.; Zhang, D.-W.; Tie, C.; Zhou, Y.-L.; Zhang, X.-X. *Biosens. Bioelectron.* **2013**, *49*, 236−242.

(13) Zhang, D.-W.; Nie, J.; Zhang, F.-T.; Xu, L.; Zhou, Y.-L.; Zhang, X.-X. *Anal. Chem.* **2013**, *85*, 9378−9382.

(14) Zhang, H.; Hu, X.; Fu, X. *Biosens. Bioelectron.* **2014**, *57*, 22−29.

(15) Zhou, Z.; Xiang, Y.; Tong, A.; Lu, Y. *Anal. Chem.* **2014**, *86*, 3869−3875.

(16) Smith, J. E.; Griffin, D. K.; Leny, J. K.; Hagen, J. A.; Chávez, J. L.; Kelley-Loughnane, N. *Talanta* **2014**, *121*, 247−255.

(17) Zhang, F.-T.; Nie, J.; Zhang, D.-W.; Chen, J.-T.; Zhou, Y.-L.; Zhang, X.-X. *Anal. Chem.* **2014**, *86*, 9489−9495.

(18) Roncancio, D.; Yu, H.; Xu, X.; Wu, S.; Liu, R.; Debord, J.; Lou, X.; Xiao, Y. *Anal. Chem.* **2014**, *86*, 11100−11106.

(19) Hamidi-Asl, E.; Daems, D.; De Wael, K.; Van Camp, G.; Nagels, L. J. *Anal. Chem.* **2014**, *86*, 12243−12249.

(20) Wei, X.; Tian, T.; Jia, S.; Zhu, Z.; Ma, Y.; Sun, J.; Lin, Z.; Yang, C. J. *Anal. Chem.* **2015**, *87*, 4275−4282.

(21) Neves, M. A. D.; Blaszykowski, C.; Bokhari, S.; Thompson, M. *Biosens. Bioelectron.* **2015**, *72*, 383−392.

(22) Taghdisi, S. M.; Danesh, N. M.; Emrani, A. S.; Ramezani, M.; Abnous, K. *Biosens. Bioelectron.* **2015**, *73*, 245−250.

(23) Zhou, J.; Ellis, A. V.; Kobus, H.; Voelcker, N. H. *Anal. Chim. Acta* **2012**, *719*, 76−81.

(24) Wang, J.; Wei, T.; Li, X.; Zhang, B.; Wang, J.; Huang, C.; Yuan, Q. *Angew. Chem., Int. Ed.* **2014**, *53*, 1616−1620.

(25) Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406−3415.

(26) Rachofsky, E. L.; Osman, R.; Ross, J. B. *Biochemistry* **2001**, *40*, 946−956.

(27) Stojanovic, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2000**, *122*, 11547−11548.

(28) Soulière, M. F.; Haller, A.; Rieder, R.; Micura, R. *J. Am. Chem. Soc.* **2011**, *133*, 16161−16167.

(29) Dallmann, A.; Dehmel, L.; Peters, T.; Mügge, C.; Griesinger, C.; Tuma, J.; Ernsting, N. P. *Angew. Chem., Int. Ed.* **2010**, *49*, 5989−5992.

(30) Shlyahovsky, B.; Li, D.; Weizmann, Y.; Nowarski, R.; Kotler, M.; Willner, I. *J. Am. Chem. Soc.* **2007**, *129*, 3814−3815.

(31) Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303−308.

(32) Liu, J. W.; Lu, Y. *Adv. Mater.* **2006**, *18*, 1667−1671.

(33) Liu, J. W.; Lee, J. H.; Lu, Y. *Anal. Chem.* **2007**, *79*, 4120−4125.

(34) Liu, J.; Mazumdar, D.; Lu, Y. *Angew. Chem., Int. Ed.* **2006**, *45*, 7955−7959.

(35) Baker, B. R.; Lai, R. Y.; Wood, M. S.; Doctor, E. H.; Heeger, A. J.; Plaxco, K. W. *J. Am. Chem. Soc.* **2006**, *128*, 3138−3139.

(36) Li, Y.; Qi, H. L.; Peng, Y.; Yang, J.; Zhang, C. X. *Electrochem. Commun.* **2007**, *9*, 2571−2575.

(37) Haller, A.; Souliere, M. F.; Micura, R. *Acc. Chem. Res.* **2011**, *44*, 1339−1348.

(38) Martin, C. T.; Ujvari, A.; Liu, C. *Methods Enzymol.* **2003**, *371*, 13−33.

(39) Stampfl, S.; Lempradl, A.; Koehler, G.; Schroeder, R. *ChemBioChem* **2007**, *8*, 1137−1145.

(40) White, R. J.; Phares, N.; Lubin, A. A.; Xiao, Y.; Plaxco, K. W. *Langmuir* **2008**, *24*, 10513−10518.

(41) Swensen, J. S.; Xiao, Y.; Ferguson, B. S.; Lubin, A. A.; Lai, R. Y.; Heeger, A. J.; Plaxco, K. W.; Soh, H. T. *J. Am. Chem. Soc.* **2009**, *131*, 4262−4266.

(42) Kang, K.; Sachan, A.; Nilsen-Hamilton, M.; Shrotriya, P. *Langmuir* **2011**, *27*, 14696−14702.

(43) Wang, T.; Hoy, J. A.; Lamm, M. H.; Nilsen-Hamilton, M. *J. Am. Chem. Soc.* **2009**, *131*, 14747−14755.

(44) Pennings, E. J. M.; Leccese, A. P.; de Wolff, F. A. *Addiction* **2002**, *97*, 773−783.

(45) Elsworth, J. D.; Taylor, J. R.; Jatlow, P.; Roth, R. H. *Drug Dev. Res.* **1993**, *30*, 189−200.

(46) Walter, F.; Vicens, Q.; Westhof, E. *Curr. Opin. Chem. Biol.* **1999**, *3*, 694−704.

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723

(47) Stoltenburg, R.; Nikolaus, N.; Strehlitz, B. *J. Anal. Methods Chem.* **2012**, *2012*, 415697.
(48) Nikolaus, N.; Strehlitz, B. *Sensors* **2014**, *14*, 3737−3755.
(49) Hamilton, P. L.; Arya, D. P. *Nat. Prod. Rep.* **2012**, *29*, 134−143.
(50) Ilgu, M.; Wang, T.; Lamm, M. H.; Nilsen-Hamilton, M. *Methods* **2013**, *63*, 178−187.
(51) Ilgu, M.; Fulton, D. B.; Yennamalli, R. M.; Lamm, M. H.; Sen, T. Z.; Nilsen-Hamilton, M. *RNA* **2014**, *20*, 815−824.
(52) Bosco, A.; Camunas-Soler, J.; Ritort, F. *Nucleic Acids Res.* **2014**, *42*, 2064−2074.
(53) Shi, Y.-Z.; Jin, L.; Wang, F.-H.; Zhu, X.-L.; Tan, Z.-J. *Biophys. J.* **2015**, *109*, 2654−2665.
(54) Huguet, J. M.; Bizarro, C. V.; Forns, N.; Smith, S. B.; Bustamante, C.; Ritort, F. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 15431−15436.
(55) Bizarro, C. V.; Alemany, A.; Ritort, F. *Nucleic Acids Res.* **2012**, *40*, 6922−6935.
(56) Moore, F. M. L.; Simpson, D. *Med. Lab. Sci.* **1990**, *47*, 85−89.
(57) Inaba, T.; Stewart, D. J.; Kalow, W. *Clin. Pharmacol. Ther.* **1978**, *23*, 547−552.
(58) Cone, E. J.; Sampson-Cone, A. H.; Darwin, W. D.; Huestis, M. A.; Oyler, J. M. *J. Anal. Toxicol.* **2003**, *27*, 386−401.
(59) Jufer, R. A.; Wstadik, A.; Walsh, S. L.; Levine, B. S.; Cone, E. J. *J. Anal. Toxicol.* **2000**, *24*, 467−477.
(60) Eskridge, K. D.; Guthrie, S. K. *Pharmacotherapy* **1997**, *17*, 497−510.
(61) Baselt, R. C. *J. Chromatogr.* **1983**, *268*, 502−505.
(62) Vasiliades, J. *J. Anal. Toxicol.* **1991**, *15*, 345.
(63) Paudel, B. P.; Rueda, D. *J. Am. Chem. Soc.* **2014**, *136*, 16700−16703.
(64) Dupuis, N. F.; Holmstrom, E. D.; Nesbitt, D. J. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 8464−8469.

DOI: 10.1021/acs.analchem.6b01633
Anal. Chem. 2016, 88, 7715−7723